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Anaerobes in the microbiome

Quantification, isolation and characterization of *Bifidobacterium* from the vaginal microbiomes of reproductive aged women

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ABSTRACT

The vaginal microbiome plays an important role in women's reproductive health. Imbalances in this microbiota, such as the poorly defined condition of bacterial vaginosis, are associated with increased susceptibility to sexually transmitted infections and negative reproductive outcomes. Currently, a "healthy" vaginal microbiota in reproductive aged women is understood to be dominated by *Lactobacillus*, although "atypical" microbiomes, such as *Bifidobacterium*-dominated profiles, have been described. Despite these observations, vaginal bifidobacteria remain relatively poorly characterized, and questions remain regarding their actual abundance in the microbiome. In this study, we used quantitative PCR to confirm the relative abundance of *Bifidobacterium* in the vaginal microbiomes of healthy reproductive aged women (n = 42), previously determined by deep sequencing. We also isolated and phenotypically characterized vaginal bifidobacteria (n = 40) in the context of features thought to promote reproductive health. Most isolates were identified as *B. breve* or *B. longum* based on *cpn*60 barcode sequencing. Fermentation patterns of vaginal bifidobacteria did not differ substantially from corresponding type strains of gut or oral origin. Lactic acid was produced by all vaginal isolates, with *B. longum* strains producing the highest levels, but only 32% of isolates produced hydrogen peroxide. Most vaginal bifidobacteria were also able to tolerate high levels of lactic acid (100 mM) and low pH (4.5 or 3.9), conditions typical of vaginal fluid of healthy women. Most isolates were resistant to metronidazole but susceptible to clindamycin, the two most common antibiotics used to treat vaginal dysbiosis. These findings demonstrate that *Bifidobacterium* is the dominant member of some vaginal microbiomes and suggest that bifidobacteria have the potential to be as protective as lactobacilli according to the current understanding of a healthy vaginal microbiome.

1. Introduction

Bifidobacteria were first described by Tissier in 1899, who isolated a bacterium from breast-fed infant feces and named it *Bacillus bifidus* [1]. In 1924, Orla-Jensen proposed the genus *Bifidobacterium* as a separate taxon for these organisms [2], which currently includes more than 30 species [3]. Bifidobacteria are Gram-positive, anaerobic, non-motile, non-spore forming rod-shaped bacteria, with varied branching. They belong to the *Bifidobacteriaceae* family and have high genomic G + C content (55–67 mol%) [3]. Bifidobacteria are known to colonize the human vagina, oral cavity and, more abundantly, the gastrointestinal tract (GIT) [4]. Several studies have shown their influence on human physiology and nutrition [5–9]. In newborns, bifidobacteria play an important role as one of the primary colonizers of the GIT, representing 60–91% of fecal bacteria in breast-fed infants [10,11]. This proportion decreases with age and it may represent less than 10% of the adult fecal microbiota [12,13]. Bifidobacteria provide protection from pathogens in the GIT through the production of bacteriocins [7], inhibition of pathogen adhesion [5], and modulation of the immune system [14,15]. Due to these health-promoting effects, bifidobacteria have been extensively studied as probiotics [8,16–18].

Early microbial colonization is an essential process in the maturation of the immune system [19]. This initial colonization may be affected by many factors, such as the mode of delivery (vaginal or caesarean section), feeding type (breast-fed or formula-fed), exposure to antibiotics and hygiene [20]. However, the relative contributions of maternal microbiota (gut, breast milk, vaginal) and

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environmental sources to the bifidobacteria population of the neonatal gut remain unresolved.

While *Bifidobacterium* spp. present in the gut are well described, vaginal bifidobacteria remain relatively poorly characterized, and it is not known if vaginal adaptation has resulted in distinct phenotypic features that distinguish them from gut populations. Although a healthy vaginal microbiota is defined as *Lactobacillus*-dominated, several studies have identified vaginal *Bifidobacterium*-dominated profiles in 5–10% of healthy, reproductive aged women [21–24]. Furthermore, vaginal bifidobacteria are reported to produce lactic acid and hydrogen peroxide; attributes of vaginal lactobacilli credited with maintaining homeostasis in the vaginal microbiome [25].

Culture-independent techniques are useful tools in microbiome characterization, but methods based on amplification and sequencing of 16S rRNA genes, have been reported to underrepresent *Bifidobacterium* in microbial communities [26]. The abundance of *Bifidobacterium* in the vaginal microbiota may also be underestimated due to the similarity of their 16S rRNA sequences to those of *Gardnerella vaginalis*. *G. vaginalis* is also a member of the *Bifidobacteriaceae* family and is a commonly detected microorganism associated with bacterial vaginosis (BV) [27]. The use of the *cpn*60 "universal target" (UT) region as a barcode for microbiome profiling results in better resolution of closely related species, including those within *Bifidobacteriaceae* [28], and *cpn*60 based human fecal microbiome profiles have been shown to more accurately represent *Bifidobacterium* content than a 16S rRNA based approach [26]. Previous studies of the vaginal microbiome [21] or synthetic mixtures of vaginal organisms [29] have demonstrated a strong correlation between *cpn*60 sequence read abundance and organism abundance determined by quantitative PCR. However, regardless of the target used, relative abundance of specific organisms within complex communities may not be represented accurately by methods that rely on polymerase chain reaction (PCR) amplification and its inherent biases.

Considering the lack of information about *Bifidobacterium* spp. of vaginal origin, their importance as a potential source for the neonatal gut microbiome, and their potential health-promoting effects in the vagina, a better understanding of the properties of vaginal bifidobacteria is needed. In this study, our main objectives were: 1) to apply species-specific quantitative PCR to confirm the relative abundance of *Bifidobacterium* in the vaginal microbiomes of reproductive aged women previously determined based on *cpn*60 barcode sequencing, and 2) to characterize vaginal *Bifidobacterium* isolates based on carbohydrate fermentation patterns, hydrogen peroxide production, lactic acid production, resistance to low pH and lactic acid, and susceptibility to antibiotics.

2. Material and methods

2.1. Samples and microbiome profiles

Vaginal microbiome profiles from 492 healthy women were previously published by our research group [30,31]. Profiles were created by PCR amplification and deep sequencing of the *cpn*60 UT region. Total bacterial load in each sample was also estimated as part of these studies using a SYBR Green assay based on the amplification of the V3 region of the 16S rRNA gene. The remaining vaginal swabs and DNA extracts from these studies, archived at −80 °C, were available for use in the current study.

2.2. Bifidobacterium quantitative PCR assays

Sequences with similarity to *Bifidobacterium breve, Bifidobacterium dentium* and *Alloscardovia omnicolens* (*Bifidobacteriaceae* family) that were detected at high frequency in the previously published studies [30,31] were selected as targets for quantitative PCR. Signature regions within the *cpn*60 UT unique to each target were determined using Signature Oligo software (LifeIntel Inc., Port Moody, BC, Canada) and primers were designed using Primer-blast software [32] and Primer3 [33] (Table 1).

To create plasmids for use in standard curves, target sequences were amplified from vaginal swab DNA extracts. The resulting PCR products were purified and ligated into cloning vector pGEM-T-Easy (Promega, Madison, WI) and used to transform competent *E. coli* DH5α. Insertion of the intended target sequence was confirmed by DNA sequencing. Optimal annealing temperature for each assay was determined using an annealing temperature gradient, and specificity of each primer set was confirmed by using plasmids containing *cpn*60 UT sequences from closely related species as template.

All qPCR reactions were performed in duplicate and each batch of reactions included a no template control and a standard curve consisting of serial dilutions of plasmids containing targets. Each reaction consisted of 2 μL of template DNA, 1 × iQ SYBR Green Supermix (BioRad, Mississauga, ON, Canada) and 400 nM each primer, in a final volume of 25 μL. A MyiQ thermocycler (BioRad) was used for all reactions with the following protocol: 95 °C for 3 m, followed by 40 cycles of 95 °C for 15 s, 65 °C for 15 s, 72 °C for 15 s. A dissociation curve was subsequently performed for 81 cycles at 0.5 °C increments from 55 °C to 95 °C to confirm the purity of PCR products.

2.3. Calculation of proportional abundance of Bifidobacterium in vaginal samples

Vaginal microbiome profiles (n = 492) were ranked according to the proportional abundance of *Bifidobacterium* (all *Bifidobacterium* species and *Alloscardovia* combined), based on the previously determined *cpn*60 sequence read counts [30,31].

B. breve, B. dentium and *A. omnicolens* DNA was quantified in vaginal swab DNA extracts from selected samples using the SYBR Green qPCR assays described in the previous section. Previously determined total 16S rRNA copy number per sample [30,31] was used an as estimate of total bacterial population. The ratio between $\log_{10}$ copy number of *Bifidobacterium* and 16S rRNA $\log_{10}$ copy number was used as an estimate of the proportional abundance of each target species in the selected vaginal microbiome samples.

Proportional abundance determined from deep sequencing of *cpn*60 UT amplicons (percent of sequence reads) and by quantitative PCR for each of the three targets evaluated (*B. breve, B. dentium* and *A. omnicolens*) were compared using Spearman rank correlation in IBM SPSS (Statistical Package for the Social Sciences, version 21).

2.4. Isolation of vaginal Bifidobacterium

A complete list of isolates used in the study and their sources is provided in Supplemental Table 1. Bifidobacteria were isolated from vaginal swabs, which were collected in previous studies and had been stored at −80 °C. For one group of samples (healthy pregnant and non-pregnant Canadian women from Vancouver, BC and Toronto, ON), 12 *Bifidobacterium*-dominated samples were selected based on the previous microbial profiling data [30,31]. Eluted material from these 12 vaginal swabs was plated on Columbia agar containing 5% sheep blood (CSB, BD Canada, Mississauga, ON). For the second group of samples (Adolescent women, Winnipeg, MB) [25], material from 27 vaginal swabs was plated on a Bifidus selective medium agar (BSM agar, Sigma-Aldrich, Oakville, ON); no sequence data from these microbiome samples was